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FROM BIOFLUID TO BIOMARKER: OPTIMIZING MICRORNA PROFILING BY RT-QPCR

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ABSTRACT

Circulating microRNAs (miRNAs) serve as promising liquid-biopsy indicators because of the stability of mature miRNAs in plasma and serum, even in the presence of many RNases. The analytical signal is, however, a composite result of blood collection, clotting or anticoagulation, platelet and erythrocyte carryover, storage, RNA recovery, reverse-transcription chemistry, amplification efficiency, and normalisation. This comprehensive evaluation assesses the whole analytical process from the management of whole blood/plasma/serum to RNA extraction, reverse transcription, and quantitative PCR. Contemporary data supports the use of matrix-specific standard operating procedures, clear quality control for haemolysis and platelet contamination, validated low-input extraction with stage-specific spike-ins, assay-specific reverse transcription validation, and normalisation utilising validated endogenous controls, global mean methods, or absolute quantification when suitable. U6 and RNU48 must not be regarded as universal extracellular reference standards. Digital PCR enhances precise quantification at minimal copy numbers; yet, it does not eradicate pre-analytical or reverse transcription biases. MIQE 2.0 (2025) enhances the reporting standards for assay design, efficacy, dynamic range, limits of detection/quantification, controls, and normalisation. Clinical translation requires a finalised Standard Operating Procedure, calibration particular to the matrix, orthogonal validation, and prospective external validation.

Keywords: Circulating miRNAs, Liquid biopsy, RNA extraction, Stem-loop RT, Reference miRNA, qPCR

1 INTRODUCTION

The circulating miRNAs are regulatory RNAs that measure between 18 and 24 nucleotides, found in whole blood, plasma, and serum. Their persistence in circulation suggests protection against rapid degradation by association with Argonaute-containing ribonucleoprotein complexes, lipoproteins, and other protein transporters, as well as encapsulation inside extracellular vesicles. The extracellular RNA domain has emphasised that "circulating miRNA" is not a unified molecular entity: total plasma miRNA, vesicle-associated miRNA, and protein-bound miRNA may originate from different biological sources and exhibit diverse analytical recovery characteristics.

This distinction is crucial for liquid biopsy. A plasma test may quantify a combined signal produced by circulating cell-free substances together with remaining platelets, erythrocytes, and leukocytes. Serum adds an additional complexity since coagulation triggers platelet activation and alters the released RNA reservoir. As a result, disparities between cases and controls might stem from disease biology, but they may also result from matrix selection, processing duration, platelet contamination, haemolysis, or extraction chemistry [4,5,14,36].

The analytical problem is amplified by low target abundance and low total RNA input. In plasma, conventional A260/A280 measurements can be dominated by background nucleic acids and contaminants rather than the small amount of circulating miRNA. Modern workflows therefore treat RNA recovery as a process to be monitored rather than inferred from NanoDrop concentration alone. The NCI evidence-based cfmiRNA recommendations specifically frame blood collection, processing, storage, extraction and quality assessment as a single standardized workflow [2,3]

2 PRE-ANALYTICAL VARIABLES & SAMPLE HANDLING

2.1 Matrix selection

Whole blood is appropriate when the biological question includes blood-cell miRNAs; it is generally inappropriate as a surrogate for cell-free plasma miRNA. Plasma avoids the coagulation-associated release of platelet RNA but remains vulnerable to residual platelets. Serum can show higher apparent levels for some platelet-associated miRNAs because clotting activates platelets. Comparative studies therefore show matrix-dependent expression differences and support material-specific assay calibration [4,37].

2.2 Anticoagulants

K2-EDTA is commonly preferred for circulating RNA studies because it is compatible with downstream molecular workflows and avoids the strong polymerase inhibition associated with heparin. Heparin can inhibit reverse transcriptase and/or DNA polymerase and may distort all normalization strategies, although the magnitude depends on anticoagulant concentration and workflow. Heparinase administration may salvage some tests; nonetheless, it is advisable to circumvent heparin when the research design allows for it [6,7]. Sodium citrate serves as an alternative plasma anticoagulant; nevertheless, it induces dilution and necessitates standardisation within cohorts.

2.3 Hemolysis

Erythrocytes are rich in miRNAs, especially miR-451a and miR-16, hence even little haemolysis may provide a misleading biological signal. An effective quality control technique integrates spectrophotometric absorbance at about 414 nm with a molecular haemolysis index that relies on the differential levels of an erythrocyte-enriched miRNA like miR-451a in comparison to a miRNA less susceptible to haemolysis, such as miR-23a-3p. The precise ΔCt cutoff is particular to the assay and platform and requires validation instead of being universally adopted as a standard threshold [3,4,5,31]. Recent studies indicate that the suggested biomarker miRNAs exhibit significant sensitivity to haemolysis [38].

2.4 Centrifugation and platelet depletion

A first low-speed centrifugation eliminates whole cells. A secondary expedited treatment or confirmed platelet-poor/platelet-free plasma technique reduces remaining platelets and detritus. This is not only a superficial enhancement: platelet-associated miRNAs might prevail in the observable circulating profile. Laboratories need to record centrifugation force in ×g instead of rpm, together with rotor type, braking, temperature, delay to separation, and the utilisation of a secondary clarifying stage [4,5,36].

2.5 Storage

Aliquot plasma or serum to minimize repeated freeze-thaw cycles and store at −80 °C when long-term biobanking is required. Liquid-nitrogen vapor storage may be used for stringent biorepository protocols, but the key variable is a validated, consistent process rather than a universally superior temperature. Recent stability experiments indicate that

many miRNAs are remarkably stable for hours under controlled conditions, but stability of an individual miRNA does not prove that the overall profile is unaffected by cell activation, hemolysis or matrix changes [2,3,36].

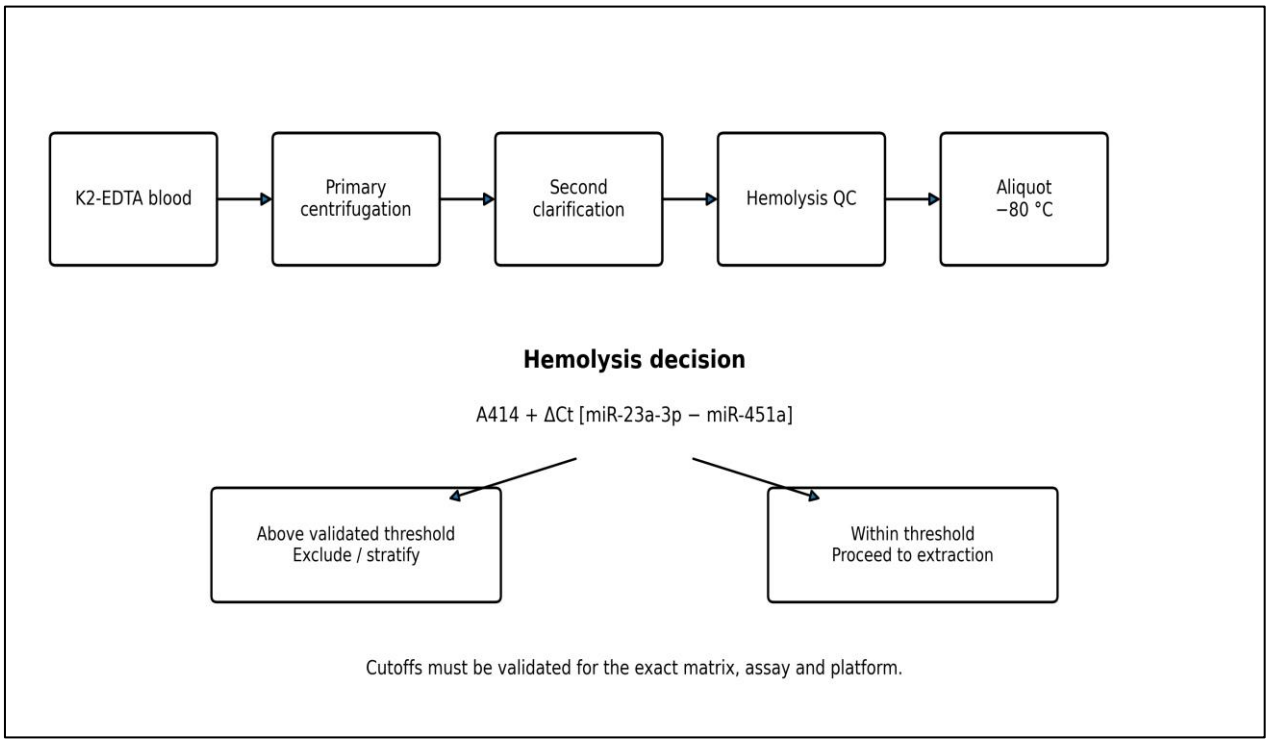

Figure 1: Pre-analytical workflow and hemolysis assessment. A practical decision architecture for cell-free miRNA studies. The hemolysis threshold must be validated for the exact matrix, assay and analytical platform.

3 RNA EXTRACTION AND PURIFICATION STRATEGIES

Organic extraction, silica spin columns, and magnetic-bead methodologies each balance recovery, purity, labour, and automation in distinct ways. TRIzol/TRIzol-LS efficiently extracts short RNAs at a low cost; nevertheless, the phase separation process relies on the operator's skill, and remaining phenol may impede subsequent operations. Silica-column kits facilitate standardisation and often provide favourable results with few plasma inputs; yet, the binding chemistry and carrier composition may include recovery bias that is depending on sequence and concentration. Magnetic beads are appealing for high-throughput labs because of their facilitation of automation and closed processes; nonetheless, the chemistry of the beads and the efficiency of washing need validation for short RNAs.

A crucial distinction is that "RNA yield" does not equate to "miRNA recovery. Plasma has little total RNA, and a standard spectrophotometer can provide an inaccurate concentration measurement. A Qubit or other fluorometric test may provide a more advantageous estimate of low-level concentrations; nevertheless, fluorometry does not verify RNA integrity or eliminate the presence of inhibitors. Bioanalyzer/TapeStation small-RNA tests can delineate size distribution when enough material is present; in instances of little plasma input, the absence of a distinct electropherogram does not always indicate unsuccessful extraction [1,2].

Enhancements in low-input recovery may be achieved by process regulation and, where substantiated, the use of carrier molecules like glycogen or MS2 RNA. Synthetic spike-ins need to be included at a specified phase—preferably before to extraction for the purpose of recovery assessment, with supplementary controls implemented before reverse transcription where it is necessary to distinguish RT efficiency from extraction efficiency. *C. elegans* miR-39/miR-54 serve as effective exogenous process controls due to their absence in human specimens; nevertheless, they do not function as biological normalisers and are incapable of adjusting for biological variations across patients [6,13,20].

EV enrichment constitutes a significant domain rather than only a standard enhancement. If the hypothesis pertains to extracellular vesicles, the separation of EVs must be well delineated and characterised; otherwise, pre-extraction enrichment of EVs may exclude non-vesicular Ago-bound miRNA and create an additional recovery bias. For a comprehensive cell-free miRNA biomarker, direct extraction of cfRNA from plasma or serum is often more indicative of the target analyte [8,14].

4 REVERSE TRANSCRIPTION METHODOLOGIES

Due to the brevity of mature miRNAs, traditional primer design is inadequate, making RT chemistry a critical factor in assay specificity. Stem-loop RT employs a target-specific structured primer to elongate the brief mature miRNA into an extended cDNA substrate. It provides significant specificity for mature miRNAs and may differentiate between closely similar sequences; nevertheless, multiplexing is limited, and each target needs a tailored RT primer.

Poly(A)-tailing approaches enzymatically add an adenine tail to miRNAs and then use an oligo(dT)-containing universal RT primer. They reduce the number of target-specific RT primers and can be convenient for profiling, but the universal step can amplify non-specific products and may be sensitive to tailing efficiency. Adapter-ligation workflows provide another strategy and are particularly useful in sequencing, but ligation efficiency can introduce sequence-dependent bias.

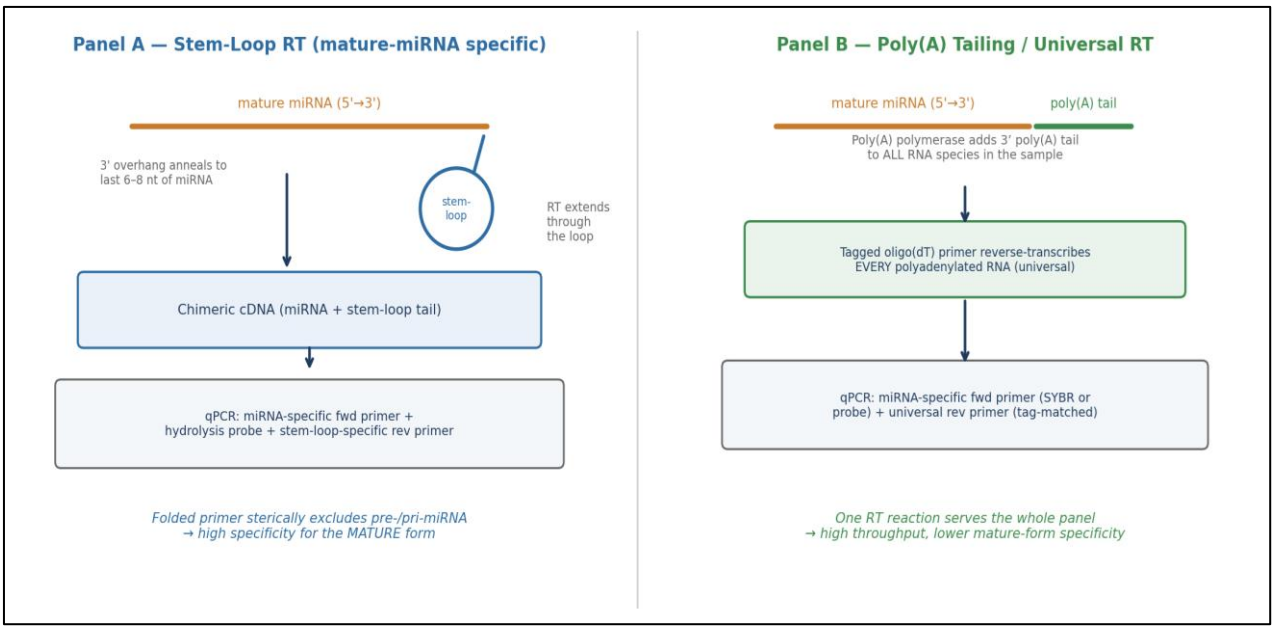

Figure 2: Molecular comparison of mature-miRNA reverse-transcription strategies. Stem-loop RT favors target specificity, whereas poly(A)-tailing provides a universal RT architecture with greater multiplexing flexibility and a greater need for specificity controls.

Pre-amplification can rescue targets near the lower end of the assay range and is especially useful for low-volume plasma profiling. However, it is itself an amplification stage and can magnify primer-specific bias. Therefore, pre-amplification should be locked during validation, applied identically to all samples and reported explicitly. The 2018 plasma qPCR-array study demonstrated that pre-amplification improved target detectability, while also showing that U6 was a poor normalizer in plasma [26].

5 RT-QPCR, CHEMISTRIES & NORMALIZATION

Hydrolysis-probe assays provide high sequence specificity and are particularly valuable for short miRNA targets. SYBR Green/EvaGreen assays are more economical and adaptable; nonetheless, they need meticulous melt-curve or amplicon validation and may be more susceptible to non-specific results. LNA-enhanced primers increase melting temperature and can improve discrimination of closely related mature miRNAs, although assay design and chemistry remain platform-specific [9,18,19].

The normalization problem remains the most serious analytical weakness of circulating miRNA RT-qPCR. U6 and RNU48 are intracellular small RNAs and are not automatically stable in plasma or serum. Their abundance can also differ from extracellular miRNAs in extraction and RT behavior. Studies of plasma qPCR arrays have specifically found U6 to perform poorly compared with algorithmic approaches [26], and broader reviews conclude that there is no universal endogenous control for all circulating-miRNA studies [10,12,14].

miR-16-5p and miR-103a-3p are sometimes used as candidate controls, but neither should be assumed invariant. miR-16 is particularly vulnerable to erythrocyte contamination, while miR-103a stability depends on matrix and cohort. A reference miRNA should be selected from the same matrix, disease spectrum and collection workflow using stability

algorithms such as NormFinder, geNorm or BestKeeper, followed by independent validation [10,12,13]. In discovery-scale profiling, global-mean normalization can be powerful when a sufficiently large and representative set of miRNAs is measured; recent plasma data support global-mean and quantile approaches in some cohorts [11].

Exogenous cel-miR-39 or cel-miR-54 is best viewed as a process control rather than a substitute for biological normalization. It can reveal extraction and RT variability, but because it is added artificially it cannot compensate for differences in circulating blood volume, platelet contamination or disease-related changes in total extracellular RNA. A strong design can therefore use both an exogenous process control and a validated endogenous/global normalization strategy [6,13,20].

Absolute quantification by digital PCR offers a different solution. Partitioning and Poisson statistics enable the direct quantification of target copies without the need of a standard curve, enhancing accuracy at low copy numbers and diminishing reliance on a relative reference. Nevertheless, dPCR does not remove pre-analytical bias, extraction loss or RT bias if an RT step is used. It should therefore be viewed as complementary to, not a replacement for, rigorous specimen control [17,20,21,22].

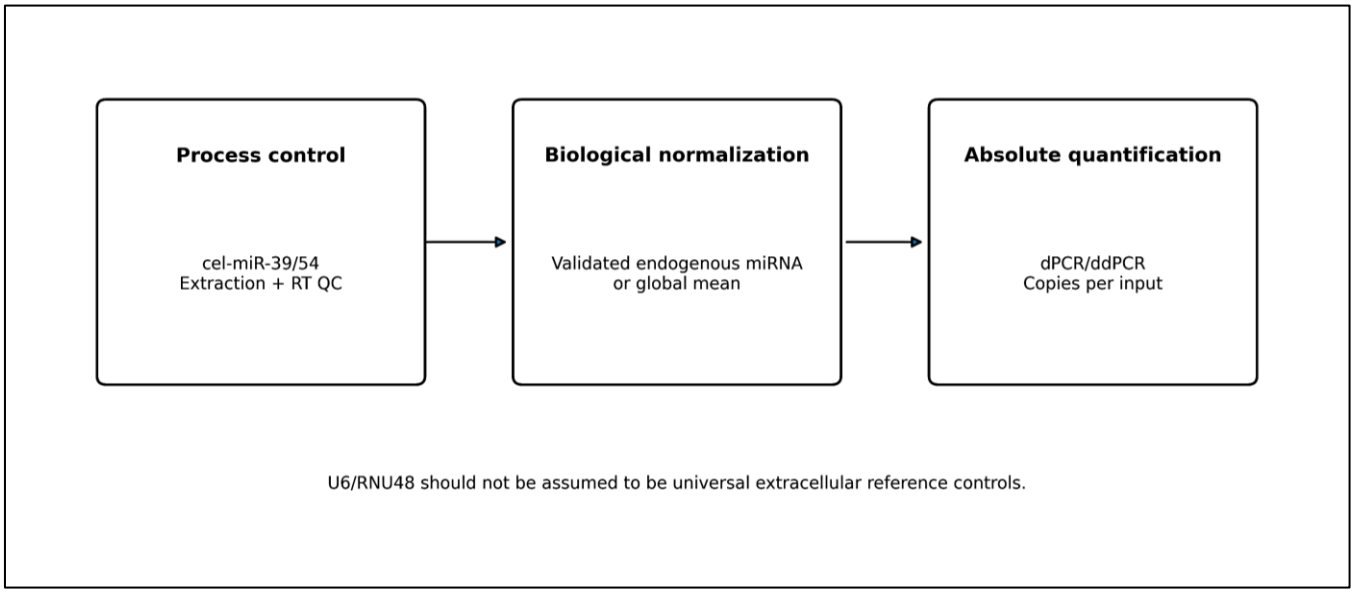

Figure 3: Normalization architecture for circulating miRNA assays. Process controls, biological normalization and absolute quantification address different sources of analytical variation and should not be treated as interchangeable.

6 ARCHITECTURE OF TABLES

The following tables summarize the practical benchmark. They intentionally avoid naming one universal winner because the optimal workflow depends on whether the target is total cfmiRNA, EV-associated miRNA or a specific clinical assay.

Table 1: Benchmarking miRNA extraction, RT and qPCR technologies

Pipeline stage	Technology	Sensitivity	Specificity	Technical pitfalls	Best practice	Key references
Sample	K2-EDTA plasma	High for cfmiRNA	High if platelet-depleted	Platelet carryover; timing variability	Rapid separation; report ×g/time; second clarification	[2–5,36]
Sample	Serum	High	Matrix dependent	Clotting changes platelet-	Serum-specific SOP; no plasma	[4,37]

				associated miRNAs	interchange without calibration	
Extraction	TRIzol/TRIzol-LS	High potential recovery	Variable purity	Phenol carryover; operator variability	Process spike-in; strict phase separation	[5,16]
Extraction	Silica spin column	High; workflow dependent	Good	Small-RNA binding/recovery bias	Validate input and elution volumes	[5,16]
Extraction	Magnetic beads	High; scalable	Good	Wash/reagent effects	Automate with fixed lots and controls	[2,3]
RT	Stem-loop	High	Very high for mature target	Target-specific primer bias; limited multiplexing	Validate every RT primer and efficiency	[9,18]
RT	Poly(A)-tailing/universal	High	Moderate–high	Non-specific products; tailing bias	No-RT/NTC controls and assay-specific validation	[9,19]
qPCR	TaqMan/MGB	High	Very high	Cost; probe/RT dependence	Efficiency, specificity and LOD/LOQ validation	[1,9]
qPCR	SYBR/EvaGreen	High	Moderate–high	Primer-dimers; non-specific products	Melt analysis and orthogonal specificity checks	[9,19]
qPCR	LNA-enhanced	Very high for close homologues	Very high	Design/platform dependence	Use validated LNA chemistry	[18,20]
Absolute	dPCR/ddPCR	Very high at low copy number	High	Partition QC; cost; upstream bias remains	Report partitions, controls, LOD/LOQ and input volume	[17,20–22]
Normalization	Validated endogenous/global mean	Context dependent	Biological relevance	No universal control	Validate in same matrix/cohort	[10–13]
Process QC	cel-miR-39/54	Tracks technical recovery	Not biological	Timing determines measured variance	Define spike-in stage explicitly	[6,13,20]

Table 2: Methodological confounders in liquid-biopsy miRNA detection

Variable	Source	Impact on profile	Mitigation strategy	Key references
Matrix	Plasma vs serum	Matrix-specific abundance; platelet-associated differences	Do not interchange matrices without calibration	[4,37]
Platelets	Residual cellular material	Inflates platelet-enriched miRNAs	Second clarification or validated platelet-poor/free plasma SOP	[4,36]
Hemolysis	RBC lysis	False elevation of miR-451a/miR-16 and other RBC miRNAs	A414 plus molecular index; predefined exclusion/stratification	[3–5,27,38]
Anticoagulant	Heparin	RT/PCR inhibition	Prefer EDTA; otherwise validate heparinase rescue	[6,7]
Processing delay	Time to separation	Cell activation/release	Standardize and record delay	[2–5,36]
Freeze–thaw	Biobanking	Handling variance; analyte-specific effects	Single-use aliquots; minimize cycles	[2–4,28,36]
Extraction	Organic/column/bead chemistry	Recovery and inhibition bias	Lock method; stage-defined process spike-in	[5,16]
Carrier	Glycogen/MS2/carrier RNA	Changes low-input recovery	Validate carrier and lot	[2,3]
RT chemistry	Stem-loop/poly(A)/adapter	Target-specific bias	Validate efficiency and specificity	[9,18,19]
Reference	U6/RNU48/miRNA controls	Normalization bias	Validate endogenous/global strategy in study matrix	[10–13,26]
Spike-in	cel-miR-39/54	Tracks process variation, not biology	Add at defined stage and report timing	[6,13,20]
Platform	qPCR/seq/dPCR	Different dynamic ranges and biases	Orthogonal validation for key biomarkers	[20–24]

7 CONCLUSION AND FUTURE PERSPECTIVES

A clinically credible circulating-miRNA assay should be implemented as a locked SOP rather than as a collection of individually optimized steps. At minimum, the SOP should define patient preparation where relevant; tube type; time from venipuncture to centrifugation; centrifugation force and temperature; platelet-depletion criteria; hemolysis QC; aliquoting; storage temperature; freeze–thaw limits; extraction input volume; spike-in timing; RT chemistry and RNA input; qPCR primer/probe concentrations; efficiency; dynamic range; LOD and LLOQ; replicate policy; normalization algorithm; exclusion criteria; and raw-data retention.

MIQE 2.0 (2025) strengthens these principles by recommending transparent reporting of assay design, RT conditions, qPCR chemistry, efficiency-corrected quantities, dynamic range, LOD/LOQ, controls, technical replicates, normalization and raw-data transparency [1]. MIQE 2.0 is not a dedicated cfmiRNA guideline; therefore, it should be combined with evidence-based specimen-processing recommendations for circulating cell-free miRNA [2,3].

Recent evidence reinforces that serum and plasma should not be treated as interchangeable matrices. A multicenter 2026 technical study comparing 202 women found matrix-dependent miRNA differences and concluded that conversion of a multi-miRNA test between serum and plasma requires experimental calibration [43]. Recent pre-analytical work also shows that hemolysis and storage conditions can materially change clinically measured miRNA concentrations [4].

The future of liquid-biopsy miRNA measurement is likely to be hybrid. Small-RNA sequencing and multiplex platforms are valuable for discovery; RT-qPCR remains attractive for targeted validation; dPCR is increasingly useful for absolute

low-copy measurement; and amplification-free platforms may eventually provide robust near-patient testing. The decisive advance, however, will be harmonized pre-analytical SOPs, matrix-specific reference materials, transparent normalization, inter-laboratory proficiency testing and prospective clinical validation.

Clinical-Translation Checklist

- Lock matrix, tube, anticoagulant and processing time before prospective validation.
- Document centrifugation in ×g, time, temperature and rotor conditions.
- Use explicit hemolysis and platelet-contamination QC.
- Validate extraction recovery with a stage-defined synthetic spike-in.
- Validate RT efficiency and assay specificity for every target.
- Do not assume U6, RNU48 or miR-16-5p is universally stable.
- Report PCR efficiency, dynamic range, LOD/LOQ, replicate rules and exclusion criteria according to MIQE 2.0.
- Use orthogonal confirmation (sequencing, dPCR or independent qPCR chemistry) for key biomarker candidates.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of conflict of interest

The authors declare that there are no conflicts of interest.

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